

FORM PTO-1390
(REV. 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

5034US

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5

09/913345

INTERNATIONAL APPLICATION NO.

PCT/NL00/00079

INTERNATIONAL FILING DATE

09 February 2000 (09.02.00)

PRIORITY DATE CLAIMED

11 February 1999 (11.02.99)

TITLE OF INVENTION

PRION TEST

APPLICANT(S) FOR DO/EO/US: Jan Gerrit GARSSSEN, Jorg Gunther JACOBS, Joannes Pieter Maria LANGEVELD
Marinus Adrianus SMITS, Lucien Johannes Mattheus VAN KEULEN, Bram Edward Cornelis SCHREUDER, Alexander BOSSEERS

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:
Copy of Preliminary Examination Report

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL740538335USDate of Deposit with USPS: August 10, 2001Person making Deposit: Blake Johnson

U.S. APPLICATION NO. 09/913345 <small>(known, see 37 CFR 1.51)</small>		INTERNATIONAL APPLICATION NO. PCT/NL00/00079		ATTORNEY'S DOCKET NUMBER 5034US	
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21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY	
				\$ 860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	16 - 20 =	0	x \$18.00	\$	
Independent claims	1 - 3 =	0	x \$80.00	\$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+	\$270.00
TOTAL OF ABOVE CALCULATIONS =				\$	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+	\$
SUBTOTAL =				\$	990.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$	990.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	
TOTAL FEES ENCLOSED =				\$	990.00
				Amount to be refunded:	\$
				charged:	\$

a. ☒ A check in the amount of \$ 990.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees.
 A duplicate copy of this sheet is enclosed.

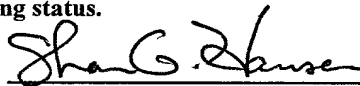
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
 overpayment to Deposit Account No. 20-1469. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card**
information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR
 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Allen C. Turner
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 SIGNATURE
 Shawn G. Hansen for
 Allen C. Turner
 NAME

42,627
 REGISTRATION NUMBER

09/913345
518 Rec'd PCT/PTO 10 AUG 2001

PATENT
5034US

#4/B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Garssen et al.

Serial No.: To be assigned

Filed: 10 August 2001

Title: PRION TEST

Examiner: To be assigned

Art Unit: To be assigned

Docket No.: 5034US

NOTICE OF EXPRESS MAILING

Express Mail Label No.: EL740538335US

Date of Deposit with USPS: 10 August 2001

Person making deposit: Blake Johnson

PRELIMINARY AMENDMENT

Hon. Commissioner for Patents
BOX NON-FEE AMENDMENT
Washington, D.C. 20231

Sir:

Prior to the calculation of fees and examination on the merits of the above-referenced application, entry of the amendments hereinafter set forth is respectfully solicited. All amendments to and cancellations of claims are made without prejudice or disclaimer.

IN THE CLAIMS:

Claims 1-7, 10-16, 18, and 19 have been amended and claims 8, 9, and 17 have been cancelled herein. All of the pending claims 1-7, 10-16, 18, and 19 are presented below in clean

form, pursuant to 37 C.F.R. §§ 1.121(c)(1)(i) and 1.121(c)(3). Applicants respectfully request entry of the claims as amended. Additionally, a version of the claims with markings to show changes made is attached hereto, pursuant to 37 C.F.R. § 1.121(c)(1)(ii).

1. (Amended) A method for reducing the risk of scoring a false-positive test result in testing at least one sample derived from a mammal for the presence or absence of an aberrant prion protein, comprising treating said at least one sample with guanidine thiocyanate or a functional equivalent thereof.
2. (Amended) The method according to claim 1, wherein said method further is for reducing the risk of scoring a false-negative test result.
3. (Amended) The method according to claim 1, wherein said at least one sample is tested in an immunoassay.
4. (Amended) The method according to claim 3, wherein said immunoassay is designed for mass-screening purposes.
5. (Amended) The method according to claim 1, further comprising treating said at least one sample with a protease to reduce the presence of normal prion protein.
6. (Amended) The method according to claim 1, wherein said mammal is a ruminant.
7. (Amended) The method according to claim 6, wherein said ruminant is ovine or bovine.

Please cancel claims 8 and 9.

10. (Amended) The method according to claim 1, further comprising:
treating at least one first sample derived from said mammal with guanidine thiocyanate or a functional equivalent thereof;

leaving at least one second sample derived from said mammal untreated with guanidine thiocyanate or a functional equivalent thereof; and
comparing the test result of said at least one first sample with said at least one second sample.

11. (Amended) The method according to claim 1, further comprising immunologically detecting said aberrant prion protein with at least one antibody directed against a proteinase K resistant part of the aberrant prion protein.

12. (Amended) The method according to claim 11, wherein said at least one antibody is directed against a proteinase K resistant N-terminal part of the aberrant prion protein.

13. (Amended) The method according to claim 11, wherein said at least one antibody is raised against a peptide derived from the aberrant prion protein.

14. (Amended) The method according to claim 13, wherein said peptide is selected from the group consisting of the amino acid sequences identified in FIG. 2 or functional equivalents thereof.

15. (Amended) The method according to claim 11, wherein said aberrant prion protein is immunologically detected in an enzyme-linked immunoassay.

16. (Amended) The method according to claim 15, wherein said enzyme-linked immunoassay comprises a dot-blot assay.

Please cancel claim 17.

18. (Amended) A kit of parts comprising means for performing the method according to claim 1.

19. (Amended) The kit of parts according to claim 18, wherein said kit of parts is designed for mass-screening purposes.

REMARKS

The foregoing amendments are intended to conform the application more closely to current United States Patent and Trademark Office practice by, for example, eliminating multiple dependencies and clarifying the claims. No new matter has been introduced by this amendment. All amendments and cancellations of claims are made without prejudice or disclaimer. Entry of the foregoing amendments is respectfully solicited.

Respectfully submitted,



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Date: 10 August 2001

Enclosures: Version With Markings to Show Changes Made

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS:

1. (Amended) [Use of guanidine thiocyanate (gdnSCN) or a functional equivalent thereof for treating at least one sample derived from a mammal]A method for reducing the risk of scoring a false-positive test result in testing [said]at least one sample derived from a mammal for the presence or absence of an aberrant prion protein, comprising treating said at least one sample with guanidine thiocyanate or a functional equivalent thereof.
2. (Amended) [Use]The method according to claim 1, wherein said method further is for reducing the risk of scoring a false-negative test result.
3. (Amended) [Use]The method according to claim 1[or 2], wherein said at least one sample is tested in [a]an immunoassay.
4. (Amended) [Use]The method according to [anyone of claims 1 to 3]claim 3, wherein said immunoassay is designed for mass-screening purposes.
5. (Amended) [Use]The method according to [anyone of claims 1 to 4]claim 1, further comprising [use of a protease for]treating said at least one sample with a protease to reduce the presence of normal prion protein.
6. (Amended) [Use]The method according to [anyone of claims 1 to 5]claim 1, wherein said [sample is derived from a ruminant]mammal is a ruminant.
7. (Amended) [Use]The method according to claim 6, wherein said ruminant is ovine or bovine.

Please cancel claims 8 and 9.

10. (Amended) [A]The method according to claim 1,[8 or 9] further comprising:
treating at least one first sample derived from said mammal with guanidine thiocyanate[gdnSCN]
or a functional equivalent thereof;
[and]leaving at least one second sample derived from said mammal untreated with guanidine
thiocyanate[gdnSCN] or a functional equivalent thereof; and
comparing the test result of said at least one first sample with said at least one second sample.

11. (Amended) [A]The method according to claim 1,[anyone of claims 8 to 10] further
comprising immunologically detecting[immunological detection of] said aberrant prion protein
[using]with at least one antibody directed against a proteinase K resistant part of the aberrant
prion protein.

12. (Amended) [A]The method according to claim 11, wherein said at least one antibody is
directed against a proteinase K resistant N-terminal part of the aberrant prion protein.

13. (Amended) [A]The method according to claim 11,[or 12] wherein said at least one
antibody is raised against a peptide derived from the aberrant prion protein.

14. (Amended) [A]The method according to claim 13, wherein said peptide is selected from
the group[s]consisting of the amino acid sequences identified[listed] in [figure]FIG. 2 or
functional equivalents thereof.

15. (Amended) [A]The method according to claim 11,[anyone of claims 11 to 14] wherein
said aberrant prion protein is immunologically detected in an enzyme-linked immunoassay.

16. (Amended) [A]The method according to claim 15, wherein said enzyme-linked
immunoassay comprises a dot-blot assay.

Please cancel claim 17.

18. (Amended) [A test kit having been provided with]A kit of parts comprising means for performing [a]the method according to claim 1[anyone of claims 8 to 17].

19. (Amended) [A test kit]The kit of parts according to claim 18[which], wherein said kit of parts is designed for mass-screening purposes.

09/913314

518 Rec'd PCT/PTO 10 AUG 2001

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#3/a

In re application of

Giancarlo BEDETTI

PCT/IB00/00145

Appln. No.: not yet assigned

Attorney Docket Q65608

Confirmation No.: not yet assigned

Group Art Unit: Not yet assigned

Filed: August 10, 2001

Examiner: Not yet assigned

For: SECONDARY REFORMING PROCESS AND BURNER

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 1, after the title, delete the heading "DESCRIPTION" and insert the following paragraph:

--Cross-Reference to the Related Application


This application is entitled to the benefit of the priority based on the International application no. PCT/IB00/00145. This application is a CIP continuation of PCT/IB00/00145, filed February 10, 2000 which claims priority on European patent application no. 99200369.9 filed February 10, 1999 and U.S. Provisional application no. 60/119,793 filed February 11, 1999.--

PRELIMINARY AMENDMENT
Attorney Docket Q65608
PCT/IB00/00145

REMARKS

Entry and consideration of this Amendment are respectfully requested.

Respectfully submitted,



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Date: August 10, 2001

PRELIMINARY AMENDMENT
Attorney Docket Q65608
PCT/IB00/00145

APPENDIX

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

The specification is changed as follows:

Page 1, after the title, delete the heading "DESCRIPTION" and insert the following paragraph:

Cross-Reference to the Related Application

This application is entitled to the benefit of the priority based on the International application no. PCT/IB00/00145. This application is a CIP continuation of PCT/IB00/00145, filed February 10, 2000 which claims priority on European patent application no. 99200369.9 filed February 10, 1999 and U.S. Provisional application no. 60/119,793 filed February 11, 1999.

Title: Prion test

5 The invention is related to diagnostic methods for detecting transmissible spongiform encephalopathies (TSEs) such as BSE, scrapie and related diseases in animals and humans.

10 Bovine spongiform encephalopathy (BSE or mad cow disease) of cattle and scrapie of sheep are fatal, non-inflammatory neurodegenerative diseases caused by prions and are characterized by a long incubation period. In humans Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia and kuru
15 belong to this category of TSEs.

20 Although scrapie, the prototype of the family of TSEs, in sheep and goats has been known for over 200 years (Pattison, 1988) and has been diagnosed world-wide (with the exception of New Zealand and Australia), it is only since 1986 that BSE has been described in cattle in the UK. By January 1998, there had been 170,259 confirmed cases of BSE in Great Britain and there may exist a great number of cases of not yet overt ("silent") BSE. BSE probably emerged because scrapie-contaminated sheep offal had been included in cattle
25 feeding-stuff via meat and bone meal and newly infected cattle material was then recycled (Wilesmith et al., 1991). This mechanism is quite plausible since ovine scrapie could be transmitted experimentally to several animal species, including cattle (Hourrigan, 1990; Gibbs, 1990).
30 Alternatively, recycling of offal from a rare case of spontaneous BSE for cattle feedstuff could also have led to the BSE epidemic. Moreover, the number of cattle in the UK with BSE reported annually is declining after the ban on feeding meat and bone meal in 1988.

Brain homogenates from cows with BSE produce after inoculation of mice a characteristic pattern of brain lesions in mice. Also characteristic incubation periods in inbred lines of mice are seen. This is identical to the pattern elicited by brain tissue from individuals who recently have died from new-variant Creutzfeldt-Jakob disease (nvCJD; Bruce, 1997). The conclusion is that the BSE agent is identical to the nvCJD agent. Up to now, this variant has caused the death of 35 young Britons and one Frenchman (Will et al, 1996; info: CJD Statistics per 30 November 1998, Internet).

There is also concern that the BSE strain that seems to be transmissible to humans may have infected sheep, where it could produce a disease hardly distinguishable from scrapie. When its ominous strain-specific properties are maintained across the species barrier, sheep BSE may be a threat to human health, although scrapie by itself seems not to transmit to humans. Indeed, BSE agent has been transmitted experimentally to sheep by the oral route (Foster et al., 1993) and thus could have the potential to infect sheep under field conditions. With the exception of a bioassay in mice, no diagnostic method is available to discriminate between BSE and scrapie in sheep at present.

Thus far, the only known component of the infectious prion is an abnormal, disease-causing isoform of the "normal" prion protein (PrP) called PrP^{Sc} or aberrant prion protein. PrP, or normal prion protein, is ubiquitous in mammalian cells in a benign, cellular conformation (PrP^C) and is encoded within a single exon as a protein of about 250 amino acid residues (figure 1). The PrP gene has been cloned and sequenced from a variety of species and there is a high degree of structural and organisational homology between mammalian PrP sequences (Schatz et al., 1995). PrPs in many mammals have a 22-24 residues long N-terminal signal sequence

as well as a 22-24 residues long C-terminal signal sequence for attachment of a GPI-anchor. This glycosyl-phosphatidylinositol linkage is a fairly common means of anchoring proteins to membranes of eukaryotic cells. Further structural characteristics of the mature protein (of 206-210 amino acid residues) are one disulfide bond and two sites for Asn-linked glycosylation.

PrP^{Sc} originates from the normal cellular isoform (PrP^C) by a post-translational process since the amino acid sequence of PrP^{Sc} is identical to that predicted from cDNA or genomic nucleic acid sequences. Glycosylation patterns are also identical between PrP^C and PrP^{Sc}. Moreover, Caughey & Raymond (1991) demonstrated that PrP^{Sc} is made from a cell surface precursor that is identical to the normal PrP. PrP^{Sc} differs from the normal, membrane bound cellular prion protein by its relative protease resistance. Treatment with proteinase K (PK) for instance, results in complete proteolysis of PrP^C whereas in PrP^{Sc} the N-terminal part is removed before the amino acid at position 90 (human numeration). The protease-resistant core left is designated PrP27-30 after its electrophoretic behavior in SDS-PAGE as a protein molecule with M_r = 27-30 kDa, and this molecular species retains full infectivity.

Further distinguishing features of PrP^{Sc} are its thermal stability, a strong tendency to aggregate and insolubility in non-denaturing detergents, apparently connected with a different molecular structure. All attempts to identify a post-translational chemical modification that features in the conversion of PrP^C into PrP^{Sc} have been unsuccessful.

The lack of a molecular explanation for the observed differences between PrP^{Sc} and PrP^C led to the proposal that they must differ in conformation. Indeed, Fourier transform infrared spectroscopy detected a content of 43% of β -sheet and 30% of α -helix structure for purified hamster PrP^{Sc} and

an even higher β -sheet content of 54% for PrP²⁷⁻³⁰. On the other hand a low content of β -sheet structure and a high α -helix content of 42% was found in PrP^C, suggesting differences in secondary structure between the aberrant and normal forms of PrP (Pan et al., 1993).

Due to its better solubility and the availability of recombinant forms of PrP^C, the three-dimensional structure of mouse PrP(121-231), involving three α -helices and a short antiparallel β -sheet, could be established by NMR (Riek et al. 1996). In the mature murine PrP^C(23-231), this segment seems to have the same fold (Riek et al., 1997). Also the spatial structure of recombinant hamster PrP(29-231) has been examined (Donne et al., 1997).

A species barrier for prion infection has been convincingly documented and found to vary widely depending on the pair of species involved and the direction of transmission. A structural basis for this species barrier is theoretically related to part or all of the amino acid replacements between the PrP of a given pair of species (Billeter et al., 1997).

Within species, genetic polymorphism in the PrP gene has been found for example with mice, humans and sheep. In sheep amino acid substitutions in PrP at a few different positions were found to correlate with different predispositions for the development of scrapie (Laplanche et al., 1993; Hunter et al., 1994; Belt et al., 1995; Bossers et al., 1996).

Studies of scrapie in goats and mice demonstrated reproducible variations in disease phenotype (length of incubation times and pattern of vacuolation) with the passage of prions in genetically inbred hosts (Bruce & Fraser, 1991). The distinct varieties or isolates of prions were called "strains". Safar et al. (1998) made plausible that the biological properties of prion strains are enciphered in the conformation of PrP^{Sc} and that strains represent different

conformations of PrP^{Sc} molecules. Infection of Syrian hamsters with eight different hamster-adapted scrapie isolates produced PrP^{Sc} molecular species which, isolated from brains in the terminal stages of disease, differed with
5 respect to protease resistance and unfolding behavior under denaturing conditions. Differences in glycosylation have also been proposed as "strain-specific" properties (Collinge et al., 1996).

Animals and humans lack a TSE disease-specific immune
10 response and TSE diagnosis is based mainly on histopathological examination, which relies on the observation of neuronal degeneration, grey matter vacuolation (the spongiform change) and astrogliosis. A distinguishing feature of TSEs is the accumulation of aberrant protein
15 (PrP^{Sc}) in the brain under continuing biosynthesis of the normal cellular PrP^C. Species differences exist however, since the relative accumulation of PrP^{Sc} in brains of hamster and mouse is approx. 10x as high as in the ruminant. Unlike the normal PrP^C, PrP^{Sc} can aggregate into amyloid-like fibrils
20 and plaques and is a major component of brain fractions enriched for scrapie activity. Therefore, a more specific diagnosis of TSEs is detection of PrP^{Sc} either in situ e.g. by immunohistochemistry or in tissue homogenates e.g. by Western blot.

25 Several poly- or monoclonal antibodies to PrP have been described. The antisera were raised in mice, hamsters, rabbits and PrP null mice and as immunogens, peptides (as linear epitopes), purified and formic acid treated PrP^{Sc} from mice, hamster or sheep and recombinant PrP are being used.
30 However, except for one case (Korth et al., 1997), there have no antibodies been developed which can discriminate between native forms of PrP^C and PrP^{Sc}, and such antibodies cannot likely discern the difference between prion strains.

By Western blotting or immunohistochemistry PrP^{Sc} could be detected in sheep in brain, spleen, tonsil or lymph node material and even in a preclinical stage of scrapie (Schreuder et al., 1998). However, in BSE infected cattle PrP^{Sc} could not be detected outside the central nervous system, not even when clinical symptoms were present.

The intriguing mechanism of prion replication is not fully understood. According to the prevailing theory, the infectious PrP^{Sc} acts as a template in the replication of nascent PrP^{Sc} molecules. In other words PrP^{Sc} imposes its own conformation upon the cellular form PrP^C or an intermediate form. A thus far unknown protein X may function as a molecular chaperone in this formation of PrP^{Sc} (Prusiner et al., 1998).

Because of the connection between BSE and the nvCJD, and the possible transfer of BSE to other species including sheep, there is a need to monitor slaughter cattle and sheep for the presence of aberrant prion protein before the meat and meat products enter the human and animal food chain or into pharmaceuticals prepared for human and animal use. Mass screening of sheep and cattle should also be of help in view of eradication programmes of scrapie and BSE. Moreover, human blood and blood products may form a health threat on account of possible contamination with blood of CJD patients and the recent occurrence of the nvCJD. For these monitoring purposes a detection method for aberrant prion protein has to be developed which should be both fast, sensitive, reliable and simple.

Bioassays for PrP^{Sc} in which different doses of the analyte are administered to target animals, are generally regarded a gold standard but otherwise are cumbersome and costly. Moreover, their quantitative character is limited by a high variation. Immunohistochemical (IHC) approaches are

very useful insofar the presence of the analyte is directly made visible in the infected tissue. In particular testing said sample by histology or cytology allows a morpological comparison of healthy and diseased cells or tissue. Also the
5 presence of PrP^{Sc} can be indicated in a preclinical phase. However, and in general histological or cytological methods are not quantitative, and hardly applicable on a large scale.

For the diagnosis of TSEs founded on the demonstration of PrP^{Sc} in infected tissues and for the assessment of PrP^{Sc}
10 itself, several methods have been described and all are on an immunochemical basis. Most of these tests have been developed and used for research-like purposes, for instance in order to quantify PrP^{Sc} during purification procedures. In some cases calibration was with recombinant PrP (hamster or mice) or
15 with PrP^{Sc}, purified from scrapie-infected brains. Otherwise, responses were expressed as a function of mg tissue equivalents; in this way also sensitivity could be assessed by the minimum amount of tissue required for the PrP^{Sc} detection.

20 ELISA systems were designed for detection of PrP^{Sc}, isolated from brains of scrapie-affected mice and hamsters (Kascsak et al, 1987) and PrP^{Sc} from murine brain and spleen (Grathwohl et al., 1997). In these assays, the PrP^C fraction was beforehand removed by PK-treatment and the purified and
25 solubilised analyte was directly coated onto the microtiter plate. Solubilisation of PrP^{Sc} was by treatment with SDS or extraction with 77% formic acid, drying and resuspension in buffer (Kascsak et al. 1987). The denaturing action of formic acid was found to enhance the antibody response to PrP^{Sc}
30 considerably compared to untreated or SDS-treated material. In this ELISA rabbit antiserum to the mouse scrapie strain ME7 PrP^{Sc} was used.

Also successive solubilization of purified PrP^{Sc} by boiling in SDS, precipitation in cold methanol and sonication

in 3-4 M guanidine thiocyanate (gdnSCN) (Grathwohl et al., 1997) apparently enhanced coating-efficiency and/or epitope density under the denaturing action of gdnSCN. On the other hand, dissolving PrP^{Sc} in SDS appeared to inhibit adsorption of PrP^{Sc} onto the polystyrene microtiter plate. Although Grathwohl et al. (1997) state that their method could be a basis for a sensitive screening method for PrP^{Sc} in crude tissue extracts, their extraction and purification steps are impracticable and time-consuming (over 22 h). The sensitivity for brain tissue was such that PrP^{Sc} could be detected in 39 mg brain equivalents; the corresponding figure for spleen tissue amounted to 313 mg. Bell et al (1997), report comparative research of five research centres of in-house immunohistochemical methods for the detection of aberrant protein in CJD by histological staining of brain tissue sections. As to the use of gdnSCN, two of the five centres employ, in addition to formic acid, gdnSCN to pretreat their tissue sections to inactivate the prion agent to allow further processing of the tissues without the danger of infection. However, all over, the value of the addition of gdnSCN is questioned, and, in the opinion of one centre, it even increases background in histology. Effective decontamination of prion containing CJD material is also shown in WO98/32334.

A sandwich type of ELISA was used to monitor the bioproduction of recombinant hamster PrP(90-231), the protease resistant core of PrP^{Sc} (Mehlhorn et al., 1996). As a capture antibody the Fab fragment of mAb 3F4 was coated. This antibody was raised against hamster scrapie strain 263K and reacts with hamster, human and feline PrP. As the second antibody mAb 13A5 (to scrapie hamster PrP^{Sc}) was used. Samples from the different stages of purification were measured in this ELISA. However, the practical conditions under which PrP^{Sc}, in order to be detected as an antigen, is

brought into an unfolded state by chaotropic agents like 3-4 M gdnSCN, are not compatible with the immunochemistry of a sandwich type of ELISA.

Prusiner et al. (1990) used an enzyme-linked immunofiltration assay (ELIFA) which combines the properties of an immuno-dot blot and ELISA technique. By this method both PrP^C and PrP^{Sc} in scrapie brain homogenates of hamsters could be quantified against a standard curve of known amounts of purified hamster PrP27-30 (0.06-4 ng). Brain homogenates, diluted in buffer with 1 M gdnSCN and 0.05% Tween 20, were applied in 5 µl quantities to nitrocellulose membrane in a manifold filtration unit. Sequential steps for immunocomplex formation with mAb 13A5 and conjugation of enzyme were also done on this membrane. For detection, dots were cut out with a puncher and placed into a microtiter plate in which color was developed. Under these conditions, immunoreactivity of the dissociated and (partly) unfolded PrP^{Sc} is indistinguishable from that of PrP^C and in this way total PrP was measured. For the determination of the PrP^{Sc} fraction, the homogenate was treated with PK prior to the ELISA and PrP^C content was calculated by subtracting the PrP^{Sc} from the total PrP.

Oesch et al. (1994) refined this ELIFA method. Samples were applied on nitrocellulose filters in the ELIFA apparatus, procedures hereafter among which a 2h-preincubation in 4 M gdnSCN to render the aberrant protein sensitive to protease digestion, and substrate binding to mAb 13A5, up to and including binding with the enzyme were done on the membrane taken out of the apparatus. For detection, membranes were placed back in the ELIFA apparatus and reacted with substrate solution. Finally, the reaction mixture was pulled through into an ELISA plate placed underneath and colour development was measured. This whole procedure took over 20 hours.

Immuno-dot blotting was used by Serban et al. (1990) for the post mortem diagnosis of Creutzfeldt-Jakob disease in humans, scrapie in sheep and scrapie-infected hamsters and mice. Direct spotting of a rather impure analyte on e.g.

5 nitrocellulose filters instead of adsorption of a purified fraction of it onto the plastic surface of microtiter wells produces a more robust ELISA variant. This qualitative test was based on the intensified immunoreactivity of PrP^{Sc}-containing amyloid plaques after treatment with 3 M gdnSCN and the protease resistance of the PrP^{Sc} isoform.

10 Brains were extracted in detergent-containing lysis buffer and 4 µl amounts were spotted onto nitrocellulose membranes. Immunoreactivity of the spotted material after successive treatment with PK and 3 M gdnSCN was conclusive
15 for the presence of PrP^{Sc} and confirmation of CJD and scrapie. Rabbit Ab R075 (to purified hamster PrP27-30) was able to detect PrP in the above four species. Out of a total of 28 human brain samples, 9 cases found positive by this method were also either defined as CJD or GSS by both
20 clinical diagnosis and a histopathological examination. For two cases, found positive by the blot procedure, histopathologic results were not available. The negative results of histopathology for CJD or GSS on the remaining 17 cases, coincided also with no indication for PrP^{Sc} with
25 the immuno-dot blot assay. In 12 histologically confirmed cases of natural scrapie in sheep, PrP^{Sc} was detected with the immunoblotting technique in the brains of 11 sheep. There are variations in the distribution of PrP^{Sc} in the brain of scrapie-affected sheep, since PrP^{Sc} was found in the spinal
30 cord, cerebellum and pons/medulla of 2 sheep, but one sheep also had PrP^{Sc} in the frontal and occipital cortex and the thalamus. This means that sampling of brain tissue could lead to false negatives due to regional variations in PrP^{Sc} content. The detection limit of this method for brain

extracts of scrapie-infected hamsters and mice ranged from 5-132 mg tissue equivalents, because these amounts still gave clearly visible spots. The duration of the test was, apart from an overnight incubation step, 6h.

5 Safar et al. (1998) developed a conformation-dependant fluorescent-ELISA that can discern various prion strains of hamsters. The assay detects a region of PrP^{Sc} that while exposed in normal PrP^C, becomes folded in the PrP^{Sc} molecule. Eu-labeled mAb 3F4 that reacts with a region of PrP^{Sc} only
10 after unfolding in 4 M gdnHCl and heating at 80°C for 5 min, was used in this assay. The immunoreactivity of the antibody to the denaturated region, as reflected by the fluorescence signal, is much higher than it is to PrP^{Sc} in its native form. The authors developed an algorithm which takes into
15 account that the immunoreactivity of antibody to denatured PrP in a sample of an affected brain is the summation of enhanced immunoreactivities of PrP^{Sc} and PrP^C during the transition from the native to the denaturated states. Knowledge of the enhancement of immunoreactivity for PrP^C during
20 denaturation was a prerequisite for this approach. For this purpose calibration curves with different concentrations of purified PrP^C were constructed. It appeared that also PrP^C showed an enhanced immunoreactivity in 4 M gdnHCl compared to its native state, albeit in a moderate way ($\leq 1.8x$). From the
25 algorithm and the measurements of a native as well as a denatured sample, the content of PrP^{Sc} could be calculated. Although this method was validated for the determination of hamster brain, the authors aim at using it also for the detection of other mammalian prions, including human. In
30 order to improve the detection threshold of the assay they introduced an initial step to selectively precipitate PrP^{Sc} from raw material with sodium phosphotungstate. In combination with this sample pretreatment, the final sensitivity of the assay could be made high. The sensitivity

limit is less than or equal to 1 ng/ml (100 pg) of PrP^{Sc}. The test however, is still far from lending itself to large-scale use in view of too much labour and long incubation times.

Capillary electrophoresis was adapted by Schmerr et al. (1995, 1996, 1998a) as a diagnostic, immunochemical assay for scrapie. The authors claim a high sensitivity (approx. 135 pg PrP^{Sc}) of their test by measuring laser-induced fluorescence of a PrP-derived fluorescein-labeled peptide after its separation by free zone capillary electrophoresis. In a preceding competition step, this peptide was displaced from a preformed complex of the peptide and an antibody directed to the unlabeled peptide in competition with the analyte (PrP^{Sc}). Beforehand, PrP^C had been removed from the analyte solution by PK-treatment. The concentration of rabbit antiserum for complex-preformation was chosen so that the antibody would be limiting in the assay (adjustment to 50% of the maximum amount of immunocomplex). Four anti-(prion)-peptide antisera were prepared and evaluated. Assays using antisera to the peptides spanning mouse amino acid position 142-154 and 155-178, differentiated scrapie-positive sheep from normal animals. In spite of the high sensitivity of this method, sample processing is time-consuming (approx. 24h) and cumbersome since PrP^{Sc} from brain stem has to be concentrated and purified through steps like ultracentrifugation and HPLC.

Western blotting (WB) in combination with SDS-PAGE is also a suitable technique for diagnosis of TSEs and a variety of different extraction procedures and Western blotting methods has been described (Race et al., 1992; Beekes et al., 1995).

Usually, PrP^C is extracted from tissues with detergents that solubilize this membrane-bound protein in a mixed micelle. However, PrP^{Sc} in the presence of detergents, aggregates and therefore is not solubilized but can be spun down by ultracentrifugation. PrP^{Sc}-aggregates dissociate in monomers

under the denaturing conditions of heating in SDS solution with β -mercaptoethanol. In this way PrP^{Sc} is electrophoretically (SDS-PAGE) indistinguishable from PrP^{C} , unless a preceding treatment with PK has been applied. This
5 proteolytic treatment removes PrP^{C} and leaves $\text{PrP}27-30$, the truncated form of PrP^{Sc} .

Race et al. (1992) could find PrP^{Sc} in every brain of 8 sheep that were histologically positive for scrapie and even in brains of clinically positive sheep that were not
10 diagnosed as scrapie-positive by histology. For detection anti-peptide antibodies to residues 89-103 and 218-232 of the mouse PrP sequence were used. Apparently, the amount of tissue required to visualise PrP^{Sc} varied among sheep from <2 to 200 mg equivalents of brain tissue. Also PrP^{Sc} was
15 found in spleens and lymph nodes in 7 of 8 sheep that had the protease-resistant form detected in brain homogenates.

One method based on WB was officially approved by the European Union (EU) and the World Organisation for Animal Health (OIE) for BSE and scrapie diagnosis (Bradley et al.,
20 1994). A minimum amount of 2 mg equivalent of infected scrapie brain allows detection of the $\text{PrP}27-30$.

Above identified assays have never been used in large screening efforts for the detection of aberrant prion protein neither in animals nor in humans.

25

Thus far, two commercial assays have been announced. In 1997 the Swiss company Prionics Inc. launched its "BSE Western Test" intended for mass screening of slaughter cattle. A modified and optimised Western blot method was used
30 to detect the proteinase K-resistant $\text{PrP}27-30$ in bovine brain stem. For immunodetection mAb 6H4 was used, developed by immunizing PrP -null mice with recombinant bovine PrP . This antibody recognizes residues 147-155 of the bovine sequence as a linear epitope in native PrP^{C} and denatured PrP^{Sc} ; this

sequence is also recognised in sheep, human, pig and mouse. Incubation with anti-mouse IgG coupled to alkaline phosphatase and detection of the enzymatic product by chemiluminescence were the final steps of the assay. This
5 test requires an incubation step with PK and detects PrP27-30. Reliability is strengthened by the Western blot documentation of the decrease in size (internal control) of the prion protein from 30-33 to 27-30 kDa. The test can be done within hours and the expectation is that subclinical BSE
10 in post-mortem brains may be detected.

Also in 1997 the Irish Company Enfer Scientific Ltd. announced the development of a BSE post-mortem test. This immunoassay intended for mass screening uses a PrP antipeptide antiserum to detect PrP27-30 in samples of brain
15 tissue of cattle after removal of PrP^C by PK-treatment. Immunodetection was enhanced by chemiluminescence. Their claims are a result within 4 hours after receipt of samples and a capacity of 14,000 cattle a day and moreover, the catching of asymptomatic animals.

20 However, these two commercial tests, although claiming high sensitivity in detecting the aberrant protein, and thus claiming to have a low number of false-negative results, suffer from the low specificity associated with the claimed high sensitivity. When using the above tests one therefore
25 runs an increased risk of falsely identifying a negative sample as false-positive, thereby falsely identifying an animal as positive. For example, Switzerland slaughtered herds in which one or more cases of BSE had been confirmed. The "Swiss reference laboratory for animal TSE" examined the
30 brains of these 1761 apparently healthy cattle by an immunohistochemical method for signs of BSE and six positive cases were detected. Also Prionics Inc. tested these 1761 cattle brains by their "BSE Western Test". Four positive outcomes were identical to the ones found by the reference

laboratory, the other two were indicated as negative and moreover two other cattle were found positive by Western blotting. Thus a total of eight positive reactors were found, four of which overlapped. These eight were re-examined in the
5 laboratory of Dr Kretzschmar (University of Göttingen) and in addition to the four undisputed cases, one of the two questionable cases identified by the reference laboratory could be confirmed (info: New Scientist, 1998, July 4 and Internet). Prionics for example scored 0.1% false-positives,
10 indicating that in 1 of every thousand cases a sample causes a false-alarm due to false-positivity.

Tests scoring false-positive results (being in general not specific enough) have other consequences than tests scoring false-negative results (being in general not
15 sensitive enough).

False-negative means that an in essence positive sample from a positive individual is scored negative, and thus is not suspected of having a TSE while in truth said individual is having a TSE. A false-negative diagnosis thus results in
20 missing positive cases.

For humans, false-negative means that no diagnosis of TSE is made where said human actually has a TSE. This causes a wrong prognosis being established and wrong treatment being given, until a second test is done.

25 For animals, especially in those cases where slaughtered animals are tested, false-negative means that no diagnosis of TSE is made where said animal was actually infected and possibly capable of spreading the disease without having been noticed. Meat and other products from such a false-negative
30 animal may contain aberrant prion protein. Such meat and meat products will be traded and eaten, and can thus be a source for further infection, notably of humans who even falsely trust that the animal has been tested well and the meat or meat product bears no risk.

False-positive means that an in essence negative sample from a negative individual is scored positive, and thus is at least suspected of having a TSE while in truth said individual is not having a TSE at all, but possibly another
5 condition.

For humans, false-positive means that a false diagnosis of TSE is made, here again resulting in false prognosis, and in faulty treatment. If said individual is not treated well as a consequence of the mis-diagnosis, his or her possible
10 other disease condition (the symptoms of which for example gave rise to the decision to test for TSE) receives no proper treatment.

For animals, false-positive means that a false diagnosis of TSE is made, however, since TSEs are notifiable diseases
15 that in general are met with strict eradication measures, said animal shall, at least in most Western countries be killed and destroyed. Furthermore, the herd from which said animals originated runs the same risk of being destroyed when the diagnosis is not corrected. For the slaughterhouse it
20 might mean that special laborious decontamination actions have to be implemented which mean temporary interference of use of the facilities and thus considerable loss of productivity. Additionally, the country where said animal or herd is falsely diagnosed for having a case of TSE among its
25 animals will be met with export restrictions. It goes without saying that, especially when said country has no (present) reported cases of TSE, such a false-positive diagnosis is highly detrimental for said countries position on foreign markets for animal products.

30 Understanding the above risks associated with false-negative or false-positive diagnoses becomes even more complicated when one understands that in general the level of false-positives scored by a diagnostic method or test is inversely related to the number of false-negatives scored by

the same test. It is an old diagnostic truth that, in many instances, a very sensitive test (having low numbers of false-negatives) cannot be very specific (and thus has a relative high number of false-positives) and vice versa.

5 However, and especially for mass screening tests that do not comprise histology or cytology, and wherein many samples need to be tested, tests having both high sensitivity and specificity are desired.

10 The invention provides use of guanidine thiocyanate (gdnSCN) or a functional equivalent thereof for treating at least one sample derived from a mammal for reducing the risk of scoring a false-positive test result in testing said sample for the presence or absence of aberrant prion protein, 15 in particular in testing said sample in a method other than histology or cytology. Using guanidine thiocyanate or its functional equivalents allows reduction of the signal arising from the normal prion protein (PrP^c) so that, for example when a gdnSCN-treated sample is compared with an untreated 20 sample, the PrP^c signal is greatly reduced. See for example Figure 3 herein describing the reduction of the signal from a PrP^{Sc} negative sample obtained by a use according to the invention. In a preferred embodiment, the invention provides use of guanidine thiocyanate (gdnSCN) or a functional 25 equivalent thereof for treating at least one sample derived from a mammal for reducing the risks of scoring both a false-positive test result or a false-negative test result in non-histologically or non-cytologically testing samples for the presence or absence of aberrant prion protein. In a preferred 30 embodiment, the invention provides use according to the invention in an immunoassay. The invention provides a reliable, simple and fast method, comprising use of gdnSCN or a functional equivalent thereof in a method for diagnosis of TSE being both highly specific as well as highly sensitive.

The invention also provides a method for reducing the risk of scoring a false-positive test result in testing a sample derived from a mammal for the presence or absence of aberrant prion protein comprising treating at least one sample with gdnSCN or a functional equivalent thereof. Because of its simplicity and, due to its non-histological nature, speed, a method according to the invention particularly lends itself to mass screening purposes of e.g. post-mortem tissues in the slaughter-line of ruminants such as cattle and sheep, but it is equally suitable in testing samples derived from other ruminants or experimental animals. In the human field the method could be used for e.g. screening lymphoid tissues and blood-derived products. Essentially, samples from all tissues, body fluids (e.g. blood, liquor) and faeces can be used.

BSE or subclinical (silent) cases of BSE can for example be detected in samples automatically taken from the brain at the time that the heads are cut off from the slaughter-animals' trunk. The method can also be used in preclinical stages during the development of scrapie, since tonsils which can be taken from the living animal, are proven to be an indicator tissue for preclinical scrapie and to contain PrP^{Sc} (Schreuder et al., 1998).

With scrapie in sheep as a model, we developed a method to unambiguously distinguish PrP^{Sc} from PrP^C. This can be done on the basis of immunodetection of PrP^{Sc} without the need of a preceding elimination of PrP^C by enzymatic proteolysis (Figure 3).

In a modified embodiment, the invention provides use according to the invention comprising use of a protease for treating said sample to reduce the presence of normal prion protein (Figure 4). This design is for example suited for the detection of aberrant prion protein in BSE.

Immunologically the signal of PrP^{Sc} can be enhanced in the presence of chaotropic agents. This enhancement is undoubtedly effected by dissociation of the polymeric PrP^{Sc} into oligo- or monomeric units, resulting in an increase of the number of epitopes available for the antibody. Moreover, the epitopes may be better exposed to the antibody through the protein defolding action of the denaturant.

Furthermore, the invention provides a method further comprising treating at least one first sample with gdnSCN or a functional equivalent thereof and leaving at least one second sample untreated with gdnSCN or a functional equivalent thereof and comparing the test result of said first sample with said second sample. We can for example discriminate between TSE-positive and negative cases after duplicate dot blotting of extracts of brain tissue onto a membrane. Extraction is in detergent-containing (lysis) buffer. One aliquot is left untreated and the other one is treated in 4 M gdnSCN (Figure 3) or, after PK-digestion, treated in 4 M gdnSCN (Figure 4). Then, after immunostaining, the treated sample is compared to the untreated sample. The signal of the normal protein (PrP^C) is greatly reduced or retains the same intensity upon treatment and can be further reduced by protease K digestion. In this way comparison of untreated and treated samples leads to a decrease or no increased of signal of samples from normal individuals but to a significant increase of signal of samples from TSE-affected animals. By this dual internal control the discriminating value of the test is considerably enforced.

The invention also provides a method comprising immunological detection of said aberrant prion protein using at least one antibody directed against the aberrant protein, preferably directed against a proteinase K resistant part of the aberrant prion protein, for example wherein said antibody is directed against a proteinase K resistant N-terminal part

of the aberrant prion protein. Monoclonal or polyclonal antibodies can be used, possibly said antibody is raised against a peptide derived from the prion protein, for example wherein said peptide is selected from an N-terminal group consisting of residues 94-111 (like 94-105 and 100-111), a C-terminal group consisting of residues 222-234 and a group consisting of residues 145-177 (sheep numbering) or sequential homologues of the PK-resistant part of PrP^{Sc} (Figure 2) or functional equivalents thereof.

The invention also provides a method according to the invention wherein said protein is immunologically detected in an enzyme-linked immunoassay, for example wherein said enzyme-linked immunoassay comprises a dot blot assay.

Also, the invention provides a test kit having been provided with means for performing a method according to the invention. This kit for example contains a carrier matrix for spotting sample extracts from tissues, organs, cells or body fluids (e.g. nerve-tissue, blood cells, etc), buffers, solutions of gdnSCN and PK, primary antibody, enzyme-labeled second antibody and enzyme-substrate. In a most preferred embodiment, said method or test kit is designed for mass-screening purposes.

The invention is further described in the detailed description herein without limiting the invention.

FURTHER DETAILED DESCRIPTION

MATERIALS AND METHODS

Phosphate buffered saline (PBS), pH 7.2 contained 136.89 mM NaCl, 2.68 mM KCl, 8.10 mM Na₂HPO₄ and 2.79 mM KH₂PO₄ in water.

PBTS: 0.2% (w/v) Tween-20 in PBS.

Two extraction buffers were used:

(a) 10 mM phosphate buffer, pH 7.0, 0.15 M NaCl and 0.25 M sucrose, used by Pan et al. (1992) to prepare microsomal fractions;

5 (b) lysis buffer (Collinge et al., 1996) consisted of 0.5% (w/v) Tergitol (type NP-40, nonylphenoxy polyethoxy ethanol, Sigma NP-40) and 0.5% (w/v) deoxycholic acid, Na-salt (Merck) in PBS, pH 7.2.

10 Guanidine thiocyanate (gdnSCN, purity > 99%; Sigma G 9277) solutions of 4 M were made up in water (pH 5.8).

Alkaline phosphatase-conjugated goat anti-rabbit IgG (GAR/AP) was from Southern Biotechnology Ass. (ITK,
15 Diagnostics B.V., Uithoorn).

Substrate for alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; tablets; Sigma B5655).

20 Usually, after PrP extraction, protease inhibitors were added to the extracts. (Complete, protease inhibitor cocktail tablets; Boehringer Nr. 1697498, Mannheim, Germany).

25 Proteinase K (EC 3.4.21.14, 20 units/mg lyophilisate Nr. 745723) and Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride Nr. 1585916) were also from Boehringer. Incubation conditions for PrP-extracts with PK were 50 µg/ml enzyme for 30 min at 37°C. In order to stop
30 this enzymatic reaction, the incubation mixture was made 1 mM in Pefabloc added from a 100 mM stock solution of the inhibitor in water.

As a blocking agent nonfat dry milk (Protifar, Nutricia) was used.

A number of hydrophilic (14) and hydrophobic (5) membranes were tested as carrier matrix. A most successful
5 representatives, polysulphone or nitrocellulose membrane types were selected. Three membrane types were routinely used: nitrocellulose (NC) membrane with a 3 mm screen (Protran BA 85/21; 0.45 mm Nr. 405891) was from Schleicher & Schuell GmbH (Dassel, Germany), Immobilon-P,
10 (polyvinylidenedifluoride, PVDF) from Millipore B.V. (Etten Leur) and Zeta-Probe (quaternary amine-nylon membrane) was from BioRad.

An Ultra-Turrax T25 mixer with a 10 mm shaft (Ika Labortechnik GmbH, Staufen, Germany) was used to homogenize
15 brain tissue. The shaft was decontaminated in 1 M NaOH.

Water of 'Milli-Q' (Millipore) quality was used throughout.

20 *Primary antibodies*

These were intentionally designed for scrapie diagnosis. Antisera were induced in rabbits using synthetic peptides with sequences (12-mers) based on the sequence of ovine PrP protein. The sequences have such differences with the rabbit
25 PrP sequence that they induce not only antibodies which recognise these peptides but also the authentic PrP protein. Other animal species like mouse which have sequence differences could be as well suitable. The sequences used for immunisation were selected 12-mers from the protease K-
30 resistant domain of PrP^{Sc}. The selected 12-mer sequences represent domains that have a low tendency to form secondary structure (α -helix or β -sheet). The antisera are reactive in diagnostic dot blotting but also in Western blotting of both PrP^C and PrP^{Sc}, in ELISAs with as coated antigens the above

peptides or PrP protein, and in immunohistochemical detection. With the peptide derived from the ovine prion protein sequence 94-105, antisera R521 and R522 were produced in rabbits. Likewise sequence 100-111 yielded antisera R504, R505, R593, R594, R595, R596 and sequence 145-177 antiserum R532. The sequence 126-143 (ovine and bovine) gave rise to antiserum R568 while sequence 223-234 (ovine and bovine) yielded antisera R523 and R524. Peptides were synthesized and used to raise anti-peptide antisera in rabbits following previously published procedures (Van Keulen et al., 1995). Antisera were confirmed to be specific for sheep PrP (both undigested and after proteinase K treatment) on Western blots of partially purified prion protein from scrapie-affected sheep brain.

Sheep samples (brain stem, cervical spinal cord) were from scrapie-affected sheep, diagnosed by histopathological and immunohistochemical examination of the brain and from normal healthy sheep (Van Keulen et al., 1995). Samples from BSE-diagnosed cattle (histopathology, immunohistochemical examination and Western blotting) were from the cervical spinal cord or brain stem.

Procedure for immuno-dot blotting: 0.5 g portions of brain tissue were cut down with a scalpel and homogenized with an Ultra-Turrax mixer (20.000 rpm/15 sec) in 4.5 ml of ice-cold lysis buffer. The homogenates were centrifuged at 1000xg for 10 min or used without centrifugation as crude homogenate. If appropriate, an aliquot of the homogenate was incubated with PK at 37°C for 30 min after which the reaction was stopped with Pefabloc (1 mM). Otherwise a cocktail of protease inhibitors was immediately added to the homogenate. Suitable dilutions of the turbid supernatants or crude homogenates in lysis buffer were spotted in 1-3 µl amounts onto two blotting membranes and left for 15 min. One membrane was incubated in

4 M gdnSCN for 10 min, the other membrane was left untreated. Washing of the treated membranes was for 10 min in PBS on a rocking platform.

5 Membranes were blocked with 5% (w/v) Protifar in PBS for 1h at 20 °C and washed in PBTS with 1% (w/v) Protifar for 5 min at 20°C. A 1-2h incubation with the primary antibody (1/1000 diluted in PBTS) at 20°C was followed by three washing steps in PBTS for 5 min each. Next, the membranes were incubated with AP-conjugated goat anti-rabbit IgG
10 (1/1000 diluted in PBTS) for 1-2 h at 20°C and washed in PBTS three times for 5 min. Substrate solution was added and the reaction was stopped with water.

RESULTS

15

DETECTION OF ABERRANT PRION PROTEIN IN SCRAPIE

Extraction efficiency for PrP^{Sc}

20 After homogenizing brain stem tissue of a scrapie-affected sheep in extraction buffer (a) or in (b) (= lysis buffer) and low-speed centrifugation which yielded supernatant 1, aliquots of this supernatant were again centrifuged at a higher speed (11,000xg, 10 min: 'high speed' supernatant 1). The loose pellets left from the first
25 centrifugation step were adjusted with buffer to the original volume, re-extracted and centrifuged at 1000xg, which yielded a supernatant 2 and a loose pellet. In addition, aliquots of all fractions were treated with PK.

30 1 µl extracts (diluted 1, 1/10 and 1/100x in their respective buffers) were spotted onto NC and immunodetection was with R522-7, an antiserum that has proven to detect ovine PrP (Van Keulen et al, 1995).

For lysis buffer the highest signal intensity was obtained for the supernatant 1. Compared to the results for

lysis buffer, the signals for extraction buffer (a) were lower for all fractions, except for the pellet. For fractions of the lysis buffer, decreased intensities were observed after pretreatment with proteinase K, especially for
5 supernatant 2, which indicates that this fraction is relatively enriched with PrP^C.

We observed dramatically intensified signals for the lysis buffer extracts, when these were diluted in 4 M gdnSCN. For supernatant 1, even after a 100-fold dilution, the signal
10 was clearly visible, which means that these scrapie brain stems PrP can be made visible in a tissue equivalent of 1 µg.

Divergent signal enhancement for PrP^{Sc} and PrP^C

Investigation of brain stem extracts of a scrapie-
15 negative sheep in lysis buffer revealed, even in a 80-fold dilution, clear signals of PrP^C. However, after pretreatment which PK no signal could anymore be observed. Surprisingly, instead of applying this PK-treatment, dilution of tissue extract in 4 M gdnSCN led also to a dramatic decrease of
20 signal intensity for PrP^C.

Next, instead of diluting lysis buffer extracted samples in 4 M gdnSCN, we applied serial dilutions of brain extracts of scrapie-positive and negative sheep in duplicate on NC membranes and incubated one membrane in 4 M gdnSCN for 10 min
25 while the other one was left untreated.

Immunodetection revealed that we easily could discriminate between scrapie positive (PrP^{Sc} and PrP^C) and scrapie negative (PrP^C) samples: a higher intensity with 4 M gdnSCN compared to an untreated sample means scrapie
30 positive, while a lower intensity with gdnSCN means scrapie negative.

This finding is the basis for a rapid and simple diagnostic test for TSEs. In this test there is in general no need for a preceding removal of PrP^C from the negative

sample.

Alternative denaturants and antisera

As an alternative for gdnSCN we investigated the effects of other chaotropic agents. After dot blotting 3 μ l dilutions of extracts of scrapie positive and negative brain stems, separate NC membranes were incubated for 10 min in chaotropic agents. The solutions used were: 4 M gdnSCN, 7.2 M urea, 4 M KSCN, 1 M thiourea, NaOH (pH 11) in water and 98% formic acid; besides one membrane was left untreated as a blank. Results for immunodetection after KSCN and thiourea did not differ from the blank. Urea induced a slight increase for the scrapie positive material and formic acid enhanced the intensity to the level of gdnSCN although this acid caused considerable shrinking of the NC membrane. Optimum enhancement with PVDF as a carrier was achieved by using 50% formic acid, no membrane shrinkage was than observed. NaOH (pH 11) on the other hand increased the signal for scrapie-negative material.

Treatment with 4 M gdnSCN turned out to be the best discrimination between scrapie positive and negative tissue samples. Moreover, this effect appeared to be pH-invariant since solutions of 4 M gdnSCN at pH 4 and 7 (in 50 mM phosphate buffer), pH 6 (in water) and pH 9 (in 50 mM carbonate buffer) gave identical results.

Five classes of antipeptide antisera to linear epitopes of sheep PrP sequences (94-105, 100-111, 126-143, 145-177 and 223-234) were examined. For comparative reasons all sera were used in a 1/500 dilution in PBTS. Antisera to the 94-105 sequence (R521, R522) and to the 100-111 sequence (R 505) proved to have the best differentiating power. On the other hand, with the antisera R568 and R532 to the sequences 126-143 and 145-177 respectively, no immunoenhancing effect of 4 M gdnSCN on PrP^{Sc} could be detected.

Blotting membranes.

Comparison of results on NC membrane with those on Zeta-Probe showed for the latter a strong aspecific coloring of the entire membrane and consequently quaternary amine-nylon as a carrier was unsuitable. On the other hand, compared to nitrocellulose a stronger adsorption for PrP was shown for the PVDF membrane (Immobilon-P).

10 DETECTION OF ABERRANT PRION PROTEIN IN BSE

From brains of BSE-positive cattle, obtained from The Netherlands, the UK, Ireland, Belgium and Switzerland and of Dutch BSE-negative cattle (diagnosed by histopathology and immunohistochemical examination), brain stems were extracted with lysis buffer in the same manner as for sheep and the low-speed supernatant 1 was used for further examination. Brain stem extracts from confirmed scrapie-negative and positive sheep were used for comparison. Aliquots of extracts were also treated with proteinase K and 3 µl amounts of dilutions in lysis buffer of PK-treated and untreated extracts were spotted onto NC membranes. Immunodetection was with 1/1000 dilutions of antisera to the 12-mer sequences 94-105 (antiserum R521), 100-111 (R505, R595, R596), 223-234 (R523, R524) and to the longer sequences 126-143 (R568) and 145-177 (R532).

Highest immunoreactivity was shown with antisera R505 and R595. After incubation with 4 M gdnSCN signal intensity of BSE-negative samples diminished; however, the immunoenhancing effect of 4 M gdnSCN on PrP^{Sc} in BSE-positive samples did not reach a comparable high level as for sheep PrP^{Sc} in scrapie. Surprisingly, antisera R523, R524 and especially R532 showed stronger immunoreactivity with bovine PrP^{Sc} than with PrP^C. Immunoreactivity of antisera R521 and R568 with bovine PrP

was very poor. No signal was obtained with the PK-treated material of BSE- and scrapie negative animals. PVDF showed a higher adsorption than NC membranes since immunostaining could be observed at higher dilutions on PVDF. The detection limit of the test with sheep recombinant PrP spotted on PVDF and using antiserum R521 or R595 is about 50 pg. Using other detection methods, however, will of course result in even lower detection levels. Thusfar, 29 case of BSE and 131 negative controls were examined. The performance was 100% (Table 1).

The design of one of our tests is that of a dot blot immunoassay which has an intrinsically higher sensitivity than an analogous ELISA assay in a microtiter plate, due to miniaturization within the blot and the higher binding capacity of the matrix material (nitrocellulose, PVDF) than of a smooth polystyrene microtiter plate surface. Because of the divergent immunoreactivity of sheep PrP^C and PrP^{Sc} during denaturation, the discriminatory power for false positive samples of our test is much higher than that of the assay of Safar and coworkers: in our assay the signal for PrP^C during denaturation in 4 M gdnSCN diminishes, whereas immunoenhancement (with 4 M gdnHCl) takes place in the assay of Safar and coworkers. As distinct from the test of Safar and coworkers, there is no need to calibrate our assay, it can be performed within four hours and it lends itself to automation. Quantification will be with densitometric techniques. Other options for the design of our assay are an ELIFA-format combined with detection in solution of an enzyme-enhanced fluorescence or luminescence signal or time-resolved detection of lanthanide fluorescence.

Table 1. Performance of dot-blot immunoassay on diagnosis of BSE

	true positive ¹	true negative ²	total	%	95% confidence interval ³
positive in test	29	0	29		
negative in test	0	131	131		
totals	29	131	160		
sensitivity				100	89.7 - 100
specificity				100	97.3 - 100

¹(Immuno)histochemically confirmed cases from five different countries. ²All confirmed negative. These 131 negative controls consisted of 45 cows suspected of BSE and 86 cows from a herd with a BSE-case. Likewise, the performance in the case of scrapie with samples of sheep is 100% (5 positive and 5 negative cases). ³ According to Blyth-Still-Casella.

REFERENCES

- Beekes, M., E. Baldauf, S. Caßens, H. Diringer, P. Keyes, A.C. Scott, A.H. Wells, P. Brown, C.J. Gibbs Jr & D.C. Gajdusek (1995). Western blot mapping of disease-specific amyloid in various animal species and humans with transmissible spongiform encephalopathies using a high-yield purification method. J. Gen. Virol. 76:2567-2576.
- Belt, P.B.F.M., I. Muileman, B.E.C. Schreuder, J. Bos-de Ruijter, A.L.J. Gielkens & M.A. Smits (1995). Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. J. Gen. Virol. 76:509-517.
- Bell J.E., Gentleman, S.M., Ironside, J.W., Mccardle, L., Lantos, P.L., Doey, L., Lowe, J., Ferguson, J., Luthert, P., McQuaid, S., and Allen, I.V. Prion protein immunocytochemistry - UK five centre consensus report. Neuropath. Appl. Neurobiol. (1997) 23:26-35.
- Billeter, M. R. Riek, G. Wider, S. Hornemann, R. Glockshuber & K. Wüthrich. (1997). Prion protein NMR structure and species barrier for prion diseases. Proc. Natl. Acad. Sci. USA 94:7281-7285.
- Bossers, A., B.E.C. Schreuder, I.H. Muileman, P.B.G.M. Belt & M.A. Smits (1996). PrP genotype contributes to determining survival times of sheep with natural scrapie. J. Gen. Virol. 77:2669-2673.
- Bradley et al. (1994). European Commission. Directorate General for Agriculture. Unit for Veterinary Legislation and Zootechnics. Protocols for the laboratory diagnosis and confirmation of bovine spongiform encephalopathy and scrapie. Report from the Scientific Veterinary Committee.
- Bruce, M.E., & H. Fraser (1991). Scrapie strain variation and implications. In: Chesebro B. (ed.). Transmissible spongiform encephalopathies. Current topics Microbiol.

Immunol. 172:125-138.

- Bruce, M.E., R.G. Will, J.W. Ironside, I. McConnell, D. Drummond, A. Suttle, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Fraser & C.J. Bostock (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* 389:498-501.
- Caughey, B. & G.J. Raymond (1991). The scrapie-associated form of PrP is formed from a cell surface precursor that is both protease- and phospholipase-sensitive. *J. Biol. Chem.* 266:18217-18223.
- Collinge, J., K.C.L. Sidle, J. Meads, J. Ironside & A.F. Hill (1996). Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* 383:685-690.
- Donne, D.G., J.H. Viles, D. Groth, I. Mehlhorn, T.L. James, F.E. Cohen, S.B. Prusiner, P.E. Wright & H.J. Dyson (1997). Structure of the recombinant full-length hamster prion protein PrP(29-131): the N terminus is highly flexible. *Proc. Natl. Acad. Sci. U.S.A.* 94: 13452-13457.
- Foster, J., J. Hope & H. Fraser (1993). Transmission of bovine spongiform encephalopathy to sheep and goats. *Vet. Rec.* 133:339-341.
- Gibbs, C.J. Jr., J. Safar, M. Ceroni, A. DiMartino, W.W. Clark & J.L. Hourrigan (1990). Experimental transmission of scrapie to cattle. *Lancet* 335:1275.
- Grathwohl, K.-U. D., M. Horiuchi, N. Ishiguro & M. Shinagawa (1997). Sensitive enzyme-linked immunosorbent assay for detection of PrP^{Sc} in crude tissue extracts from scrapie-affected mice. *J. Virol. Meth.* 64: 205-216.
- Hourrigan, J.L. (1990). Experimentally induced bovine spongiform encephalopathy in cattle in Mission, Tex, and the control of scrapie. *J. Am. Vet. Med. Assoc.* 196:1678-1679.
- Hunter, N., W. Goldmann, G. Smith & J. Hope (1994). The association of a codon 136 PrP gene variant with the

- occurrence of natural scrapie. Arch. of Virol. 137:171-177.
- Ikegami, Y., M. Ito, H. Isomura, E. Momotani, K. Sasaki, Y. Muramatsu, N. Ishiguro & M. Shinagawa (1991). Pre-clinical and clinical diagnosis of scrapie by detection of PrP protein in tissues of sheep. Vet Rec. 128:271-275.
 - Kaszsak, R.J., R. Rubenstein, P.A. Merz, M. Tonna-DeMasi, R. Fersko, R.I. Carp, H.M. Wisniewski & H. Diringer (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J. Virol. 61:3688-3693.
 - Korth, C., B. Stierli, P. Streit, M. Moser, O. Schaller, R. Fischer, W. Schulz-Schaeffer, H. Kretzschmar, A. Raeber, U. Braun, F. Ehrensperger, S. Hornemann, R. Glockshuber, R. Riek, M. Billeter, K. Wüthrich & B. Oesch (1997). Prion PrP^{Sc}-specific epitope defined by a monoclonal antibody. Nature 390:74-77.
 - Laplanche, J.L., J. Chatelain, D. Westaway, S. Thomas, M. Dussausy, J. Brugere-Picoux & J.M. Launay (1993). PrP polymorphism associated with natural scrapie discovered by denaturing gradient gel electrophoresis. Genomics 15:30-37.
 - Mehlhorn, I., D. Groth, J. Stöckel, B. Moffat, D. Reilly, D. Yansura, W.S. Willett, M. Baldwin, R. Fletterick, F.E. Cohen, R. Vandlen, D. Henner & S.B. Prusiner (1996). High-level expression and characterization of a purified 142-residue polypeptide of the prion protein. Biochemistry 35:5528-5537.
 - Muramatsu, Y., A. Onodera, M. Horiuchi, N. Ishiguro & M. Shinagawa (1994). Detection of PrPres in sheep at the preclinical stage of scrapie and its significance for diagnosis of insidious infection. Arch. Virol. 134:427-432.
 - Oesch, B., M. Jensen, P. Nilsson & J. Fogh (1994). Properties of the scrapie prion protein: quantitative analysis of protease resistance. Biochemistry 33:5926-5931.
 - Pan, K.-M., N. Stahl, & S.B. Prusiner (1992) Purification and properties of the cellular prion protein from Syrian

hamster brain. Protein Science 1:1343-1352.

- Pan, K.-M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E. & S.B. Prusiner (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc. Natl. Acad. Sci. U.S.A. 90:10962-10966.
- Pattison, I.H. (1988). Fifty years with scrapie: a personal reminiscence. Vet. Rec. 123:661-666.
- Prusiner, S.B., M. Scott, D. Foster, K.-M. Pan, D. Groth, C. Mirenda, M. Torchia, S.-L. Yang, D. Serban, G.A. Carlson, P.C Hoppe, D. Westaway & S.J. DeArmond (1990). Transgenic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. Cell 63:673-686.
- Prusiner, S.B., M.R. Scott, S.J. DeArmond & F.E. Cohen (1998). Prion protein biology. Cell 93:337-348.
- Race, R.E., D. Ernst, A. Jenny, W. Taylor, D. Sutton & B. Caughey (1992). Diagnostic implications of detection of proteinase K-resistant protein in spleen, lymph nodes, and brain of sheep. Am. J. Vet. Res. 53:883-889.
- Riek, R., S. Hornemann, G. Wider, M. Billeter, R. Glockshuber & K. Wüthrich (1996). NMR-structure of the mouse prion protein domain PrP(121-231). Nature 382:180-182.
- Riek, R., S. Hornemann, G. Wider, R. Glockshuber & K. Wüthrich (1997) NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231). FEBS Lett. 413: 282-288.
- Safar, J., H. Wille, V. Itri, D. Groth, H. Serban, M. Torchia, F.E. Cohen & S.B. Prusiner (1998). Eight prion strains have PrP^{Sc} molecules with different conformations. Nature Medicine 10:1157-1165.
- Schatzl, H.M., M. Da Costa, L. Taylor, F.E. Cohen & S.B. Prusiner (1995). Prion protein gene variation among

- primates. J. Mol. Biol. 245:362-347.
- Schmerr, M.J., K.R. Goodwin, R.C. Cutlip & A.L. Jenny (1995). A competition assay to detect scrapie prion protein by capillary electrophoresis. J. Microcolumn Separations 7:521-527.
 - Schmerr, M.J., K.R. Goodwin, R.C. Cutlip & A.L. Jenny (1996). Improvements in a competition assay to detect scrapie prion protein by capillary electrophoresis. J. Chromatog. B 681:29-35.
 - Schmerr, M.J. & A. Jenny (1998). A diagnostic test for scrapie-infected sheep using a capillary electrophoresis immunoassay with fluorescence-labeled peptides. Electrophoresis 19:409-414.
 - Schreuder, B.E.C., L.J.M. van Keulen, M.E.W. Vromans, J.P.M. Langeveld & M.A. Smits (1996). Preclinical test for prion diseases. Nature 381:563.
 - Schreuder, B.E.C., L.J.M. van Keulen, M.E.W. Vromans, J.P.M. Langeveld & M.A. Smits (1998). Tonsillar biopsy and PrP^{Sc} detection in the preclinical diagnosis of scrapie. Vet. Rec. 142:564-568.
 - Serban, D., A. Taraboulos, S.J. DeArmond, S.J. & S.B. Prusiner (1990). Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. Neurology 40:110-117.
 - Van Keulen, L.J.M., B.E.C. Schreuder, R.H. Meloen, G. Mooij-Harkes, M. Poelen-van den Berg, M.E.W. Vromans & J.P.M. Langeveld (1995). Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. Vet. Pathol. 32:299-308.
 - Van Keulen, L.J.M., B.E.C. Schreuder, R.H. Meloen, G. Mooij-Harkes, M.E.W. Vromans & J.P.M. Langeveld (1996). Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. J. Clin. Microbiol. 34:1228-1231.

- Wilesmith, J.W., J.B.M. Ryan & M.J. Atkinson (1991). Bovine spongiform encephalopathy: epidemiological studies on the origin. Vet. Rec. 128:199-203.
- Will, R.G., J.W. Ironside, M. Zeidler, S.N. Cousens, K. Estibeiro, A. Alperovitch, S. Poser, M. Pocchiari, A. Hofman & P.G. Smith (1996). A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 347:921-925.

LEGENDS TO THE FIGURES

Figure 1:

Amino acid sequences of human, rabbit, hamster, mouse, cattle and sheep PrP genes. The entire amino acid sequence of human PrP is given; open spaces in the other sequences indicate identity. Polymorphisms are indicated in bold at the top of each block and relate to the shaded positions. ↓: PHGGGWGQ. |: protease-sensitive site, right of which the sequence for the PK-resistant core of PrP^{Sc} is found. The mature PrP is devoid of N and C terminal signal peptides (in huPrP: amino acids 1-22 and 232-253, respectively).

human:

- Kretzschmar, H.A., S.B. Prusiner, L.E. Stowring & S.J. DeArmond (1986). Scrapie prion proteins are synthesized in neurons. Am. J. Pathol. 122:1-5.

rabbit:

- Loftus, B. & M. Rogers (1997). Characterization of a prion protein (PrP) gene from rabbit: a species with apparent resistance to infection by prions. Gene 184:215-219.
- Rubenstein, R., R.J. Kascsak, M. Papini, R. Kascsak, R.I. Carp, G. LaFauci, R. Meloen & J. Langeveld (1998) J. Neuroimmunology (accepted).

golden Syrian hamster:

- Basler, K., B. Oesch, M. Scott, D. Westaway, M. Wälchli, D.F. Groth, M.P. McKinley, S.B. Prusiner & C. Weissmann (1986). Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell 46:417-428.

mouse:

- Locht, C., B. Chesebro, R. Race & J.M. Keith (1986). Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. Proc. Natl. Acad. Sci. USA 83:6372-6376.
- Westaway, D., P.A. Goodman, C.A. Mirenda, M.P. McKinly, G.A. Carlson & S.B. Prusiner (1987). Distinct prion proteins in short and long scrapie incubation period mice. Cell 51:651-662.

cattle:

- Goldmann, W., N. Hunter, T. Martin, M. Dawson & J. Hope (1991). Different forms of the bovine PrP gene have five or six copies of a short, g-c-rich element within the protein-coding exon. J. Gen. Virol. 72:201-204.

sheep:

- Goldmann, W., N. Hunter, J.D. Foster, J.M. Salbaum, K. Beyreuther & J. Hope (1990). Two alleles of a neural protein gene linked to scrapie in sheep. Proc. Natl. Acad. Sci. USA 87:2476-2480.

Figure 2:

Peptide sequences, derived from the prion protein structures of six species (**hu**=human, **rb**=rabbit, **ha**=hamster, **mo**=mouse, **bo**=cattle, **ov**=sheep). The amino acid sequence of the human peptides is given; open spaces in the other sequences indicate identity.

Antipeptide antibodies were raised in rabbits against the peptides of the ovine structure. Corresponding antisera are indicated **R5xx** at the top of each set of sequences.

Figure 3:

Prion test in which the extract was applied to two pieces of NC-membrane indicated: untreated and treated. A 10% (wt/vol.%) extract of brain-stem tissue was 1/3 diluted and 1 μ l applied to NC-membrane. Then, one piece of membrane was incubated in solution without gdnSCN (untreated), the other was incubated in 4 M gdnSCN-containing solution (treated). Further incubations for immunochemical visualization with first antibody (R522-7) and alkaline-phosphatase conjugate were according to standard procedures. In each 3-fold dilution series, the first (left) spot represents 33 μ g of tissue equivalents.

Figure 4:

Prion test, in which the extract was applied to two pieces of PVDF-membrane indicated: untreated and treated. For each negative and positive case, a 10% (wt/vol.%) extract of brain-stem tissue was prepared and divided in two portions, of which one was incubated with proteinase K (PK-digested extract) or not (undigested extract). Next, each of the extracts was 1/3 diluted and 3 μ l applied to PVDF-membrane. Then, the piece of membrane with the undigested extract was incubated in solution without gdnSCN (untreated), the membrane with digested extract was incubated in 4 M gdnSCN-containing solution (treated). Further incubations for immunochemical visualization with first antibody (R595-4) and alkaline-phosphatase conjugate were according to standard procedures. In each 3-fold dilution series, the first (left) spot represents 100 μ g of tissue equivalents.

UA 339

26

CLAIMS

What is claimed is:

1. A polymerization process comprising the steps of:
admixing a halogen-containing compound, monomer, a cobalt complex, and reducing agent.
2. A polymerization process comprising the steps of:
admixing a halogen-containing compound, monomer, and a cobalt complex selected from the group consisting of cobaloxime, organocobaloxime, cobalt porphyrin, and mixtures thereof, wherein the halogen-containing compound is a polymer containing at least one carbon-halogen bond having the halogen atom bound pendant to the polymer backbone, bound to an organic group that is pendent to the polymer backbone, or bound at the polymer chain end, and the cobalt complex reacts with the at least one carbon-halogen bond to form a radical capable of participating in a catalytic chain transfer or pseudo-living polymerization process.
3. A process for forming a methacrylate-functionalized polymer comprising the steps of:
admixing a halogen-containing polymer containing at least one carbon-halogen bond having the halogen atom bound pendent to the polymer backbone, bound to an organic group that is pendent to the polymer backbone, or bound at the polymer chain end, a cobalt complex selected from the group consisting of cobaloxime, organocobaloxime, cobalt porphyrin, and mixtures thereof, and a methacrylate ester, and
heating the admixture, wherein the halogen-containing compound is a polymer containing at least one carbon-halogen bond having the halogen atom bound pendant to the polymer backbone, at the polymer chain end, or both, and the cobalt complex reacts with the at least one carbon-halogen bond to form a radical capable of participating in a catalytic chain transfer process with the methacrylate ester.
4. A process for forming polyacrylate-functionalized polymer comprising the steps of:

UA 339

27

admixing a halogen-containing polymer containing at least one carbon-halogen bond having the halogen atom bound pendent to the polymer backbone, bound to an organic group pendant to the polymer backbone, or bound at the polymer chain end, a cobalt complex selected from the group consisting of cobaloxime, organocobaloxime, cobalt porphyrin, and mixtures thereof, and acrylate monomer, and

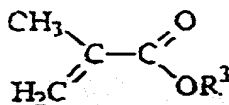
heating the admixture, wherein the cobalt complex reacts with the at least one carbon-halogen bond to form a radical capable of participating in a pseudo-living polymerization process with the acrylate monomer.

5. The process of claims 1-4, wherein said step of admixing is conducted in hexane, benzene, toluene, acetone, methylethyl ketone, tetrahydrofuran, methylcellusolve, dioxane, decalin, or mixtures thereof.
6. The process of claim 1, wherein the halogen-containing compound is a polymer containing at least one carbon-halogen bond, where the halogen atom is bound pendent to the polymer backbone, bound to an organic group that is pendant to the polymer backbone, or bound at the polymer chain end.
7. The process of claims 2, 3, 4, or 6, where the carbon in the at least one carbon-halogen bond is attached to a stabilizing group selected from phenyl groups, carbonyl groups, nitrile groups, vinyl groups, and substituted vinyl groups.
8. The process of claims 2, 3, 4, or 6, where said halogen-containing compound is selected from styrene-butadiene copolymers containing chloromethylstyrene units, brominated isobutylene-isoprene copolymers, and unsaturated polyesters containing reactive halogen atoms.
9. The process of claim 1, where said halogen-containing compound is selected from CCl_4 , CBr_4 , CBrCl_3 , $\Phi\text{CH}_2\text{Br}$, $\Phi\text{CH}_2\text{Cl}$, CCl_3CH_3 , ΦCHCl_2 , $\text{CCl}_3\text{COCCl}_3$, and CCl_3COOR , wherein R is an alkyl group, substituted alkyl group, aryl group, and substituted aryl group.

UA 339

28

10. The process of claims 1 or 2, where the monomer is a methacrylate monomer, vinyl aromatic monomer, methacrylamide, or mixture thereof.
11. The process of claim 10, wherein the methacrylate monomer is selected from methyl methacrylate, ethyl methacrylate, propyl methacrylate, t-butyl methacrylate, phenyl methacrylate, hydroxyethyl methacrylate, and N, N-dimethylmethacrylamide.
12. The process of claim 11, where the admixture contains from about 10 to about 5000 parts by weight methacrylate monomer per one hundred parts by weight halogen-containing compound.
13. The process of claims 1 or 2, further comprising the step of heating the admixture.
14. The process of claim 13, where the admixture is heated to a temperature of about 70°C.
15. The process of claims 2, 3, or 4, where said admixture further comprises a reducing agent.
16. The process of claim 15, wherein said reducing agent is selected from zinc, iron, copper, and hydrogen.
17. The process of claim 16, where said reducing agent is zinc.
18. The process of claim 1, wherein said reducing agent is selected from zinc, iron, copper, and hydrogen.
19. The process of claim 3, wherein the methacrylate monomer is:

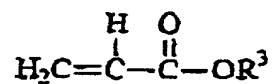


UA 339

29

wherein R includes an organic group.

20. The process of 4, wherein the acrylate monomer is:



wherein R³ includes an organic group.

21. The process of 4, where said step of admixing is conducted at a temperature from about 70°C to about 150°C.

1/4

1	hu	M A - - N L G C W M L V L F V A T W S D L G L C K K R P K P -	28
1	rb	- - - H L L A M T V - - -	29
1	ha	- - - S Y L A T M T V - - -	28
1	mo	- - - G Y L A T M T V - - -	28
1	bo	V K S H I S I M M V - - -	31
1	ov	V K S H I S I M M V - - -	31
29	hu	G G W N T G G S R Y P G Q G S P G G N R Y P P Q G G G G W G Q P	60
30	rb	- - - S - - -	60
29	ha	- - - - - - -	60
29	mo	- - - - - - -	59
32	bo	- - - - - - -	63
32	ov	- - - - - - -	63
61	hu	H G G G W G Q P H G G G W G Q P H G G G - W G	90
61	rb	- - - - - - -	90
61	ha	- - - - - - -	90
60	mo	- - - - - - -	89
64	bo	- - - - - - -	94
64	ov	- - - - - - -	94
91	hu	Q G G G G T H S Q W N K L P S K P K T N F K H M A G A A A A G A	120
91	rb	- - - - - - -	119
91	ha	- - - - - - -	120
90	mo	- - - - - - -	119
95	bo	- - - - - - -	123
95	ov	- - - - - - -	123
121	hu	V V G G L G G Y M L G S A M S R P F I I H F G S D Y E D R Y Y	150
120	rb	- - - - - - -	149
121	ha	- - - - - - -	150
120	mo	- - - - - - -	149
124	bo	- - - - - - -	153
124	ov	- - - - - - -	153
151	hu	H R E N M H R Y P N Q V Y Y R P M D E Y S N Q N N F V H D C V	180
150	rb	- - - - - - -	179
151	ha	- - - - - - -	180
150	mo	- - - - - - -	179
154	bo	- - - - - - -	183
154	ov	- - - - - - -	183
181	hu	N I T I K Q H T V T T T T K G E N S E T E T D V K M M E R V V	210
180	rb	- - - - - - -	209
181	ha	- - - - - - -	210
180	mo	- - - - - - -	209
184	bo	- - - - - - -	213
184	ov	- - - - - - -	213
211	hu	E Q M C I T Q Y E R E S Q A Y Y - Q R G - S S M - V - L F S S P P V	240
210	rb	- - - - - - -	239
211	ha	- - - - - - -	241
210	mo	- - - - - - -	241
214	bo	- - - - - - -	243
214	ov	- - - - - - -	243
241	hu	I L L I S F L I F L I V G	253
240	rb	- - - - - - -	252
242	ha	- - - - - - -	254
242	mo	- - - - - - -	254
244	bo	- - - - - - -	256
244	ov	- - - - - - -	256

Amino acid sequences of human, rabbit, hamster, mouse, cattle and sheep PrP genes. The entire amino acid sequence of human PrP is given; open spaces in the other sequences indicate identity. Polymorphisms are indicated in bold at the top of each block and relate to the shaded positions. ↓: PHGGGWGQ. | : protease-sensitive site, right of which the sequence for the PK-resistant core of PrP^{Sc} is found. The mature PrP is devoid of N and C terminal signal peptides (in huPrP: amino acids 1-22 and 232-253, respectively).

Fig. 1

Rabbit anti-peptide antisera to parts of the ovine PrP-structure

R521, R522 (to ovine sequence 94-105)

90	hu	G	Q	G	G	G	T	H	S	Q	W	N	K	P
90	rb	G			-				N			G		
90	ha	G							N					
89	mo	G							N					
94	bo	G			-				G					
94	ov	G			-	S								

R504, R505, R593-596 (100-111)

97	hu	S	Q	W	N	K	P	S	K	P	K	T	N	
96	rb	N			G								S	
97	ha	N												
96	mo	N												
100	bo	G												
100	ov													

R568 (126-143)

123	hu	G	G	L	G	G	Y	M	L	G	S	A	M	S	R	P	I	I	H
122	rb																L		
123	ha																M	M	
122	mo										M						M		
126	bo																L		
126	ov																L		

R532 (145-177)

142	hu	G	S	D	Y	E	D	R	Y	Y	R	E	N	M	H	R	Y	P	N	Q	V	Y	Y	R	P	M	D	E	Y	S	N	Q	N	N	S	
141	rb		N												Y											V		Q								
142	ha		N		W										N											V		Q			N					
141	mo		N		W										Y											V		Q								
145	bo																									V		Q								
145	ov		N												Y											V		R								

R523, R524 (223-234)

220	hu	R	E	S	Q	A	Y	Y	-	Q	R	G	-	S																						
219	rb	Q				A			-				-	A	-																					
220	ha	K							D	G		R	-	S																						
219	mo	K							D	G		R	-																							
223	bo								-				A																							
223	ov								-				A																							

Fig. 2

Fig. 3

example of test-result with brain-stem extract
from sheep

SHEEP

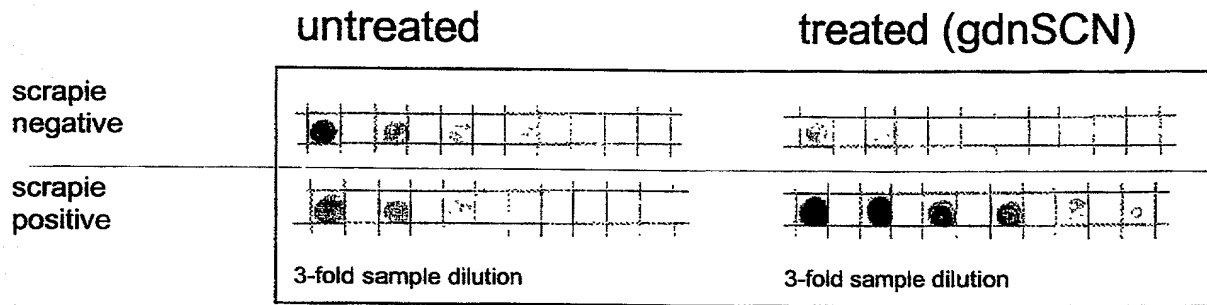










Fig. 4

example of test-result with brain-stem tissue
from sheep and cattle

CATTLE

	untreated	treated (PK, gdnSCN)
BSE negative		
BSE positive	 3-fold sample dilution	 3-fold sample dilution

SHEEP

	untreated	treated
scrapie negative		
scrapie positive	 3-fold sample dilution	 3-fold sample dilution

Power of Attorney

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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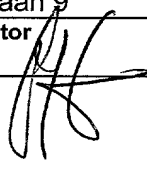
I hereby authorize them or others whom they may appoint to act and rely on instructions from and communicate directly with the person/organization who/which first sends this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instructed otherwise.

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I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below:

Prior Provisional Application(s)	
Serial Number	Day/Month/Year Filing Date
Serial Number	Day/Month/Year Filing Date
Serial Number	Day/Month/Year Filing Date

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s), or under 35 U.S.C. §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

Prior U.S. or International Application(s)		
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2/2

Declaration and Power of Attorney Patent Application (Design or Utility)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: 'Prion test'

the specification of which

- ☐ is attached hereto
- x was filed on August 10, 2001 as application serial no. 09/913,345
and or PCT International Application number PCT/NL00/00079 and was amended
on (if applicable).

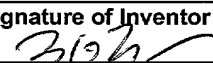
I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information know to me to be material to patentability as defined in 37 C.F.R.§1.56.

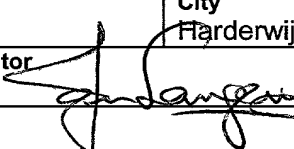
I hereby claim foreign priority benefits under 35 U.S.C.§119(a)-(d) or 35 U.S.C.§365(b) of any foreign application(s) for patent or inventor's certificate, or 35 U.S.C.§365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate of PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)		
Number	Country	Day/Month/Year Filed
99200391.3	EP	11-02-1999
Number	Country	Day/Month/Year Filed
Number	Country	Day/Month/Year Filed

2-00

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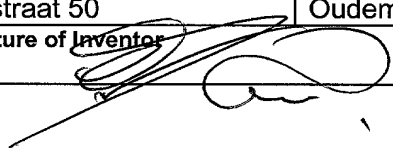
3-00

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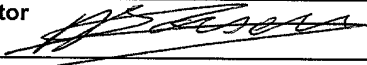
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